British Journal of Pharmacology (2006), 147, 681–689
www.nature.com/bjp

Substance P-induced cyclooxygenase-2 expression in human umbilical vein endothelial cells

*,[1]Margherita Gallicchio, [1]Arianna Carolina Rosa, [1]Elisa Benetti, [1]Massimo Collino, [1]Chiara Dianzani & [1]Roberto Fantozzi

[1]Department of Anatomy, Pharmacology and Forensic Medicine, University of Turin, Via Pietro Giuria, 9, 10125 Torino, Italy

1 Substance P (SP) is a neuropeptide involved in neurogenic inflammation and an agonist for NK_1, NK_2, and NK_3 receptors. SP induces prostaglandin (PG) production in various cell types, and these eicosanoids are responsible for numerous inflammatory and vascular effects.

2 Cyclooxygenase (COX) are needed to convert arachidonic acid to PGs. The study evaluated the effect of SP on COX expression in human umbilical vein endothelial cells (HUVEC).

3 COX-2 protein expression was upregulated by SP with a peak at 100 nM and at 20 h; in the same experimental conditions COX-1 protein expression was unchanged. A correlation between COX-2 expression and PGI_2 and PGE_2 release was detected.

4 Dexamethasone (DEX) inhibited SP-mediated COX-2 expression. Mitogen-activated protein kinases (MAPK) p38 and p42/44 were activated by SP, whereas SB202190 and PD98059, inhibitors of these kinases, blocked COX-2 expression. 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone (DFU), an experimental selective COX-2 inhibitor, blocked SP-induced PG release.

5 By RT–PCR and Western blot analysis, we demonstrated that NK_1 and NK_2 but not NK_3 receptors are present on HUVEC. Selective NK_1 and NK_2 agonists, namely [Sar^9, $Met(O_2)^{11}$]SP and [β-Ala^8] NKA(4–10), upregulated COX-2 protein expression and PG production, whereas senktide (Suc–Asp–Phe–MePhe–Gly–Leu–Met–NH_2), a selective NK_3 agonist, was ineffective in this respect. The NK_1 selective antagonist L703,606 ((*cis*)-2-(diphenylmethyl)-*N*-((2-iodophenyl)-methyl)-1-azabicyclo(2.2.2)octan-3-amine) and the NK_2 selective antagonist SR 48,968 ((S)-*N*-methyl-*N*-(4-(4-acetylamino-4-phenylpiperidino)-2-(3,4 dichlorophenyl)butyl) benzamide) competitively antagonised SP-induced effects.

6 The study shows HUVEC to possess functional NK_1 and NK_2 receptors, which mediate the ability of SP to induce expression of COX-2 in HUVEC, thus showing a previously-undetected effect of SP on endothelial cells.
British Journal of Pharmacology (2006) **147,** 681–689. doi:10.1038/sj.bjp.0706660;
published online 28 January 2006

Keywords: Substance P; cyclooxygenase-2; HUVEC; MAPKs; NK_1 receptor; NK_2 receptor

Abbreviations: COX-2, cyclooxygenase; DEX, dexamethasone; DFU, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)-phenyl-2(5H)-furanone; HUVEC, human umbilical vein endothelial cells; MAPK, mitogen-activated protein kinase; NKA, neurokinin A; NKB, neurokinin B; PG, prostaglandin; PMNs, polymorphonuclear cells; RT–PCR, reverse transcriptase–polymerase chain reaction; SP, substance P

Introduction

Substance P (SP) is a neuropeptide belonging to the tachykinin family, a group of regulatory peptides sharing the common C-terminal sequence Phe–X–Gly–Leu–Met–NH_2 (Patacchini & Maggi, 1995). Other members of this family found in mammals are neurokinin A (NKA) and neurokinin B (NKB). SP is released from unmyelinated sensory nerve endings, thus evoking inflammatory peripheral effects such as vasodilatation, plasma extravasation and leukocyte activation, which are collectively referred to as 'neurogenic inflammation' (Foreman & Jordan, 1984; Brunelleschi *et al*., 1991; Joos & Pauwels, 2000; Dianzani *et al*., 2001; Harrison & Geppetti, 2001). The effects of tachykinins are mediated by specific G-protein-coupled receptors, of which at least three subtypes have been characterised: NK_1, NK_2, and NK_3. The existence of an NK_4 receptor in man has not yet been conclusively demonstrated (Patacchini & Maggi, 1995; Page & Bell, 2002). The mammalian tachykinins display different selectivities for their receptors: SP is more selective for NK_1, NKA for NK_2, and NKB for NK_3 (Regoli *et al*., 1994). Using selective SP-receptor agonists and antagonists, we recently demonstrated that SP induces PMN adhesion to human umbilical vein endothelial cells (HUVEC) and that this effect is mediated by NK_1 and NK_2 receptors (Dianzani *et al*., 2003). In the present study, the presence of SP receptors on HUVEC is investigated by RT–PCR and Western blot techniques; the results confirm that NK_1 and NK_2 but not NK_3 receptors are present on HUVEC.

Cyclooxygenase, known also as prostaglandin H_2 synthase (PGHS), is the enzyme that catalyses the first two steps in the biosynthesis of prostaglandins (PGs) from arachidonic acid (Vane *et al*., 1998). About a decade ago, COX was shown to

*Author for correspondence;
E-mail: margherita.gallicchio@unito.it

exist as at least two distinct isoforms, COX-1 and COX-2. COX-1 is responsible for PG and thromboxane production in gastric mucosa and platelets; COX-2 is expressed constitutively in some organs, including the kidney and the brain (Vane *et al*., 1998).

HUVEC are known to possess both COX isoforms and to display, at Western blot analysis, variable basal levels of COX-2: either no band, or a weakly measurable band. This discrepancy may depend on cell isolation and culture procedures employed in different studies (Hirai *et al*., 1999; Caughey *et al*., 2001; Uracz *et al*., 2002). COX-2 induction in HUVEC has been demonstrated to occur in response to different proinflammatory cytokines, such as IL-1α and β or TNF- α (Caughey *et al*., 2001; Eligini *et al*., 2001).

SP evokes the release of PGs from various cell types, including cultured spinal cord astrocytes (Koyama *et al*., 1999; Marriott *et al*., 1991), human nasal epithelial cells (Jallat-Daloz *et al*., 2001) and HUVEC (Alhenc-Gelas *et al*., 1982; Marceau *et al*., 1989). However, in the latter study, neither COX expression nor the underlying cell mechanisms were investigated. SP has been shown to stimulate DNA synthesis in several cell types, by activating the p38 and p42/44 mitogen-activated protein kinases (MAPK); this activation is an important signalling mechanism that participates in the development of the inflammatory process (Kyriakis & Avruch, 2001; Yang *et al*., 2002). MAPKs are involved in regulating PG biosynthesis, and COX-2 induction is associated with their phosphorylation (Guan *et al*., 1998).

The study addressed the question of whether SP affects COX-2 expression in HUVEC. It investigated time- and dose-dependence of SP-evoked COX-2 expression and of PGI_2 (measured as 6-keto-$PGF_{1\alpha}$) and PGE_2 release from HUVEC challenged by SP. The pharmacological modulation of these events was evaluated using dexamethasone (DEX) to modulate gene expression of COX-2, and using 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone (DFU) as experimental selective COX-2 inhibitor. The involvement of p38 and p42/44 MAPKs was evaluated using two inhibitors of these kinases, SB202190 and PD98059. SP-receptors were identified on HUVEC by RT–PCR and Western blot techniques. The effects of SP were characterised in terms of receptors through NK selective agonists and antagonists.

Methods

Cell culture

HUVEC were isolated as has been described (Jaffe *et al*., 1973) and cultured on gelatine-coated culture dishes in M199 medium supplemented with 20% heat-inactivated bovine calf serum (BCS), 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 5 U.I. ml^{-1} heparin, 12 μg ml^{-1} bovine brain extract and 200 mM glutamine. HUVEC were utilised at II–IV passages.

Cell incubation

In time-course experiments, HUVEC were stimulated with SP 100 nM from time 0–24 h and every 4 h cells were processed for protein extraction. In dose–response experiments, HUVEC were treated with SP 1 nM–1 μM for 20 h. To analyse agonist effects, HUVEC were stimulated with SP or [Sar^9, Met(O$_2$)^{11}]SP, [β-Ala^8] NKA(4–10) or senktide (Suc–Asp–Phe–MePhe–Gly–Leu–Met–NH_2), all at 100 nM, for 20 h. To analyse the effect of SP in the presence of selective NK_1 and NK_2 antagonists, respectively L703,606 and SR 48,968, HUVEC were preincubated for 30 min with 100 nM of selective antagonist and then stimulated with 100 nM SP for 20 h. To examine the MAPK signalling pathways, HUVEC were incubated with SP 100 nM for 2, 5, 10, 20, 40, 60, and 120 min. In some experiments, HUVEC were preincubated for 30 min with either the p38 MAPK inhibitor SB202190 (50 μM) or the p42/44 MAPK inhibitor PD98059 (50 μM), washed twice and incubated with SP 100 nM for 20 h (Davies *et al*., 2000). HUVEC were also stimulated with 100 nM SP or DEX or coincubated with SP and DEX for 20 h. To inhibit COX-2 activity, HUVEC were incubated with 100 nM SP and 10 μM DFU for 20 h. Following the appropriate treatment, cells were lysed for Western blot analysis or processed for PG measurement by the enzymeimmunoassay (EIA) system as described below.

Western blotting

Cell culture dishes were washed with phosphate-buffered saline (PBS) before adding ice-cold lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA, 1 μl ml^{-1} protease inhibitors, 0.1 mM $ZnCl_2$, and 1 mM Ph–Me–Sul–Flu). Cell lysates were centrifuged at 14,000 r.p.m. for 15 min and the supernatant was recovered. The protein concentrations of cell lysates were determined using a BCA protein assay following the manufacturers' directions. HUVEC lysate samples containing 15 μg of protein were then subjected to SDS–PAGE using an 8 or 10% gel. Proteins were transferred to a PVDF membrane, which was then incubated with SuperBlock blocking buffer.

COX-2 and COX-1 proteins were detected following incubation, respectively, with a rabbit polyclonal or a mouse monoclonal antibody diluted 1 : 200 in PBS containing 0.1% Tween-20 (PBS-T) for 2 h at room temperature. Phospho-p38 or Phospho-p42/44 proteins were detected following incubation with a mouse monoclonal antibody diluted 1 : 2000 in PBS-T overnight at 4°C. SP receptors were detected following incubation with a rabbit polyclonal antibody for NK_1 or a goat polyclonal antibody for NK_2 and NK_3, diluted 1 : 200 in PBS-T for 2 h at room temperature. The secondary antibody for detection of proteins was horseradish peroxidase-conjugated donkey antirabbit IgG for COX-2 and NK_1, horseradish peroxidase-conjugated sheep antimouse IgG for COX-1, phospho-p38 and phospho-p42/44 and horseradish peroxidase-conjugated donkey antigoat for all other primary antibodies. Secondary antibodies were diluted 1 : 10,000 in PBS-T and incubated for 30 min at room temperature. To confirm the homogeneity of the proteins loaded, the membranes were stripped and incubated with β-actin monoclonal antibody (1 : 5000) and subsequently with horseradish peroxidase-conjugated sheep antimouse IgG (1 : 10,000) both for 30 min at room temperature. The membranes were overlaid with Western Lightning Chemiluminescence Reagent *Plus* and then exposed to Hyperfilm ECL film. Protein bands were quantified on film by densitometry, using the software Gel Pro.Analyser 4.5, 2000.

RNA extraction and reverse transcription

Total RNA was isolated from HUVEC with the NucleoSpin® RNA II kit, following the manufacturers' instructions. The quantity of total RNA was determined by spectrophotometric measurement at 260 nM. 1 μg of total RNA was reverse-transcribed into cDNA by incubating with 200 U RevertAid™ H Minus M-MuLV Reverse Transcriptase per 20 μl reaction at 42°C for 1 h in presence of RiboLock™ Ribonuclease inhibitor (20 U), oligo(dT)$_{18}$ primers (0.5 μg) for NK_2, NK_3, and β-actin or random hexamer primers (0.2 μg) for NK_1, dNTP (1 mM) in reaction buffer (50 mM Tris-HCl pH 8.3, 50 mM KCl, 4 mM $MgCl_2$, 10 mM DTT).

After denaturing at 94°C for 5 min, cDNA was subjected to PCR amplification. PCR mixes contained 1 μM primers, the buffer supplied by the manufacturers, 200 μM of each dNTPs, 2 mM $MgCl_2$, 1.25 U of Taq DNA polymerase, and cDNA (3–5 μl of RT–PCR reactions) in 50 μl. PCR was performed using a Tpersonal 48 Whatman Biometra thermal cycler. Positive and negative strand PCR primers used were: CAAAATGATGATTGTCGTGGTGTGC and GTGAAGA GCAGTTGGAGGTCAGGTC for NK_1 (425 bp fragment); TGCTGGTGGTGCTGACGTTTGCCATCTGCT and CTG TTGACTCTCGTGGAGAGGGAGGTCGT for NK_2 (292 bp fragment); GGCTGGCAATGAGCTCAACCATGTACAAT CCCA and GGTGAGCTTATGAAACTTGAAGTGGCGG AGGCA for NK_3 (243 bp fragment); TGACGGGGTCACCC ACACTGTGCCCATCTA and CTAGAAGCATTTGCGGT GGACGATGGAGGG for β-actin (660 bp fragment). The primers were designed by using the analysis software Primer 3 (Rozen & Skaletsky, 2000) based on their location in different exons of the genomic sequences in addition to their lack of significant homology to sequences present in GenBank (ENTREZ, BLAST, NCBI, U.S. National Library of Medicine, 8600 Rockville Pike, Bethesda, U.S.A.). Amplification of the β-actin gene transcript was used as an internal control of RT–PCR reactions among samples. The PCR cycle consisted of 95°C for 30 s, 63°C for 30 s, and 72°C for 1 min for NK_1 amplification, 95°C for 30 s, 68°C for 30 s, and 72°C for 1 min for NK_2 and NK_3 amplification and 95°C for 45 s, 60°C for 45 s, and 72°C for 90 s min for β-actin amplification. The PCR products were separated by gel electrophoresis, stained with ethidium bromide, visualized and photographed (Digital camera Canon Power Shot G6) under UV transillumination. Each PCR experiment was carried out on three different umbilical cords and controls containing no reverse transcriptase and no template were included. Amplicon size were verified by comparison with a DNA mass ladder.

Prostaglandin production

PGE_2 and the stable metabolite of prostacyclin, 6-keto-$PGF_{1\alpha}$, were measured by the EIA system following the manufacturers' instructions.

Statistical analysis

Data were expressed as means ± s.e. Statistical analysis was performed with Graphpad Prism 3.0 software. One-way analysis of variance (ANOVA), Dunnett's multiple comparison and Student–Newman–Keuls tests were used to determine significant differences between means. $P \leqslant 0.05$ was considered significant.

Materials

Medium 199, mouse monoclonal antibody against β-actin, protease inhibitors cocktail, DEX, glutamine, heparin and SP were from Sigma (Chemical Co., St Louis, MO, U.S.A.). BCS was from GIBCO BRL (Grand Island, NY, U.S.A.). The selective NK_1 agonist [Sar^9, $Met(O_2)^{11}$]SP, the selective NK_2 agonist [β-Ala^8] NKA(4–10) the selective NK_3 agonist senktide and the selective NK_1 antagonist L703,606 were from Research Biochemicals International/Sigma, (Natik, MA, U.S.A.). The selective NK_2 antagonist SR 48,968 ((S)-*N*-methyl-*N*-(4-(4-acetylamino-4-phenylpiperidino)-2-(3,4dichlorophenyl)butyl) benzamide) was a kind gift from Dr X. Edmonds-Alt (Sanofi Recherche, Montpellier, France). The MAPK inhibitors SB202190 and PD98059 were from Calbiochem (San Diego, CA, U.S.A.). The BCA Protein Assay and SuperBlock blocking buffer were from Pierce Biotechnology Inc. (Rockford, IL, U.S.A.); Polyvinyldenedifluoride (PVDF) was from Millipore (Bedford, MA, U.S.A.). Rabbit polyclonal antibody against human COX-2 and murine monoclonal antibody against COX-1 were from Cayman Chemicals (Ann Arbor, MI, U.S.A.). Rabbit polyclonal antibody against human NK_1 receptor and goat polyclonal antibodies against NK_2 and NK_3 receptors were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). Antibodies against the phosphorylated forms of p38 and p42/44 MAPKs were from Cell-Signalling (Beverly, MA, U.S.A.). Anti-mouse and anti-rabbit Ig horseradish peroxidase linked whole antibody, Hyperfilm ECL film and EIA system for PG measurement were from Amersham Biosciences Corp. (Piscataway, NJ, U.S.A.) and Western Lightning Chemiluminescence Reagent *Plus* was from PerkinElmer Life Science (Cetus, Norwalk, CT, U.S.A.). Gel Pro.Analyser 4.5, 2000 was from Media Cybernetics Inc. (Leiden, The Netherlands). NucleoSpin® RNA II was from Macherey–Nagel (Düren, Germany). RevertAid™ H Minus M-MuLV Reverse Strand cDNA Synthesis kit and Taq DNA Polymerase were from Fermentas (Harrington Court, Burlington, Ontario, Canada). All primers were synthesised and purified by MGW-Biotech (Ebersberg, Germany). All the other reagents utilised were from Sigma Chemical Co., St Louis, MO, U.S.A.

Results

Effects of SP on COX-2 expression and PG-synthesis by HUVEC

COX-2 protein was low or undetectable in nonstimulated HUVEC. Treatment of HUVEC with 100 nM SP caused COX-2 to be expressed, with the maximum increase at 20 h. COX-1 expression remained unchanged with SP treatment (Figure 1a and b). Densitometric analysis of the autoradiogram showed that SP induced a two-fold increase in COX-2 expression *versus* nonstimulated HUVEC (Figure 1a). As shown in Figure 1c, dose–response experiments gave a bell-shaped curve in the nM–μM range, with maximum effect at 100 nM. In this concentration range, SP did not alter COX-1 expression (data not shown). To analyse the

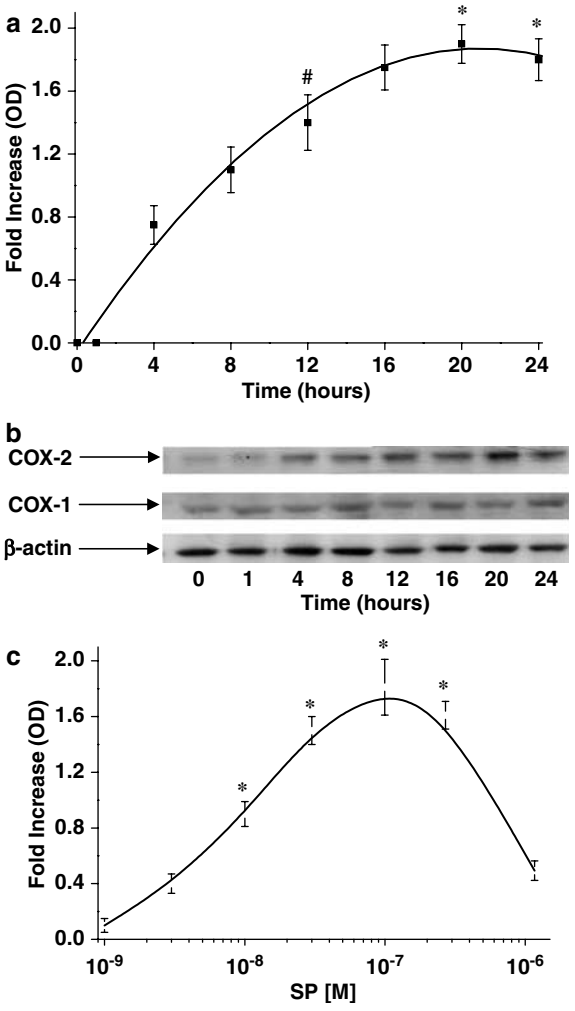

Figure 1 Time course and dose–response of COX-2 protein induction by SP. (a) HUVEC were stimulated with SP (100 nM) from time 0–24 h. At the times indicated, cells were processed for Western blot analysis as described in the Methods section and analysed by densitometry. Results are expressed as fold increase (optical density, OD) over control value (=1), which was subtracted from all experimental values (means ± s.e. of five separate experiments). Data were analysed by one-way analysis of variance (ANOVA) and the Student–Newman–Keuls test (*$P<0.01$ and $^{\#}P<0.05$ *versus* control). (b) Immunoblot of one experiment representing modulation of COX-2 protein expression by SP 100 nM. (c) HUVEC were incubated with SP 1 nM–1 μM for 20 h, and cells were subjected to Western blot analysis as described in the Methods section. Results are expressed as fold increase (OD) over control value (=1), which was subtracted from all experimental values (means ± s.e. of five separate experiments). Data were analysed by one-way analysis of variance (ANOVA) and the Student–Newman–Keuls test (*$P<0.01$ *versus* control).

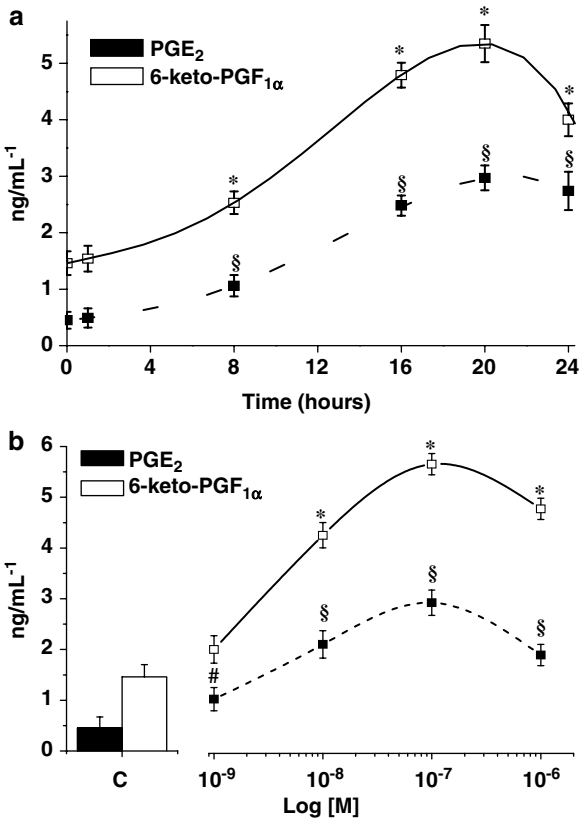

Figure 2 Time course and dose–response of PG production induced by SP. HUVEC were stimulated with SP (100 nM) from time 0–24 h (a) or with SP 1 nM–1 μM for 20 h (b). 6-keto-$PGF_{1\alpha}$ or PGE_2 were analysed for PG release determination as described in the Methods section. The results are expressed as means ± s.e. of three independent experiments performed in duplicate. Data were analysed by one-way analysis of variance (ANOVA) and the Dunnet test (*$P<0.01$ *versus* control for 6-keto-$PGF_{1\alpha}$; $^{\S}P<0.01$ and $^{\#}P<0.05$ *versus* control for PGE_2).

functionality of the expressed COX-2, we performed dose–response and time-course experiments in the above experimental conditions, and determined that SP induces 6-keto-$PGF_{1\alpha}$ and PGE_2 synthesis at the same times and concentrations utilised to obtain protein expression (Figure 2a and b). As the strongest COX-2 protein band was at 100 nM SP and at 20 h of SP incubation, we chose these two experimental conditions for all subsequent experiments.

Effect of SP on MAPK activation

We evaluated the MAPK signalling pathways that SP might activate in HUVEC. Coincubation of HUVEC with 100 nM SP resulted in the phosphorylation of both p38 and p42/44 MAPKs in a time-dependent manner (Figure 3a and b). Activation of p38 MAPK peaked at 10 min after SP addition and could still be clearly detected after 60–120 min, while p42/44 MAPK was maximally phosphorylated 5 min after SP addition, activation lasting for up to 10 min and then slowly decreasing. This activation pathway completely overlaps the activation timings of these kinases in similar experimental conditions. In the absence of SP there was little or no detectable phosphorylation of either p38 or p42/44 MAPK. To further investigate the specificity of SP-induced MAPKs activation, we utilised selective inhibitors of these two kinases. SB202190 is a pyridinyl imidazole compound that has been shown to be a specific inhibitor of p38, while PD98059 inhibits mitogen-activated protein/extracellular signal-related kinase 1 (MEK1) which is an upstream regulatory kinase of p42/44, and is known to specifically inhibit its activation (Davies *et al.*, 2000). When HUVEC were treated with SB202190 or PD98059, the SP-induced increase in COX-2 protein expression was consistently inhibited (Figure 3c); SP thus appears

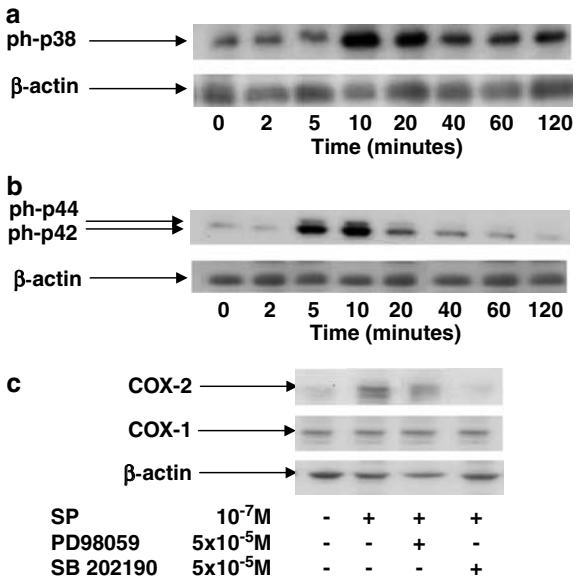

Figure 3 Effect of SP on phosphorylation of p38 and p42/44 MAPK in HUVEC. Cells were incubated with SP (100 nM) for the time indicated, from 0 to 120 min and then processed for Western blot analysis of the phosphorylated forms (ph−) of p38 (a) or p42/44 (b) as described in the Methods section. (c) HUVEC were preincubated for 30 min with either the p38 MAPK inhibitor SB202190 (50 μM) or the p42/44 MAPK inhibitor PD98059 (50 μM) followed by the addition of SP (100 nM) for 20 h. Cells were then processed for Western blot analysis as described in the Methods section. The results are representative of three separate experiments.

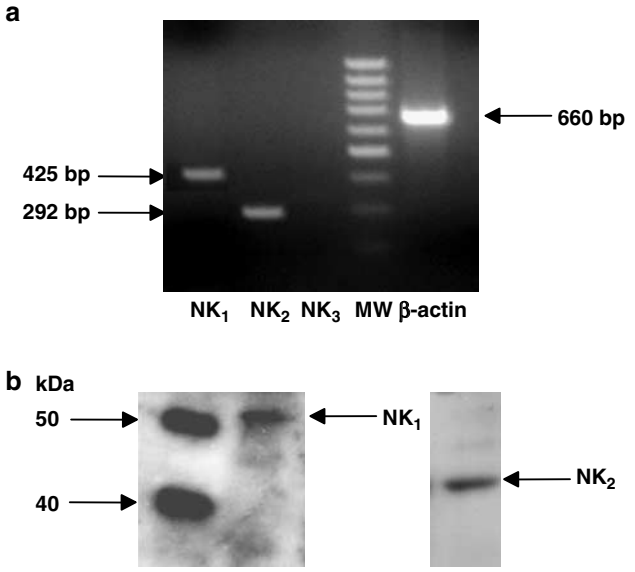

Figure 4 Identification of SP receptors on HUVEC. (a) Agarose gel showing products of RT–PCR assay for cDNA from HUVEC. Single transcripts corresponding to the sizes predicted for NK_1 (425 bp) and NK_2 (292 bp) receptors were detected. The mRNA of the NK_3 receptor was not detected after amplification of the usual quantity of cells cDNA. β-actin was used as housekeeping gene. MW, molecular size standard. Data are representative of cells freshly isolated from three different umbilical cords. (b) Western blot analysis of NK_1 and NK_2 receptors in HUVEC, showing the expected molecular weight of 53 kDa for NK_1 and 44 kDa for NK_2. The migration of protein standards of known sizes is shown on the left. Data are representative of cells freshly isolated from three different umbilical cords.

to upregulate COX-2 expression in endothelial cells through a mechanism involving both the p38 and the p42/44 MAPK pathways.

Identification of SP receptors on HUVEC

To evaluate the involvement of SP receptor(s) in COX-2 expression and PG production, we ran tests to identify the presence of NK_1, NK_2, and NK_3 receptors on HUVEC. RT–PCR revealed the presence of the first two (NK_1 425 bp, NK_2 292 bp) but not of the third (343 bp) (Figure 4a). These data were confirmed by protein analysis, demonstrating the presence of NK_1 and NK_2 proteins with the respective

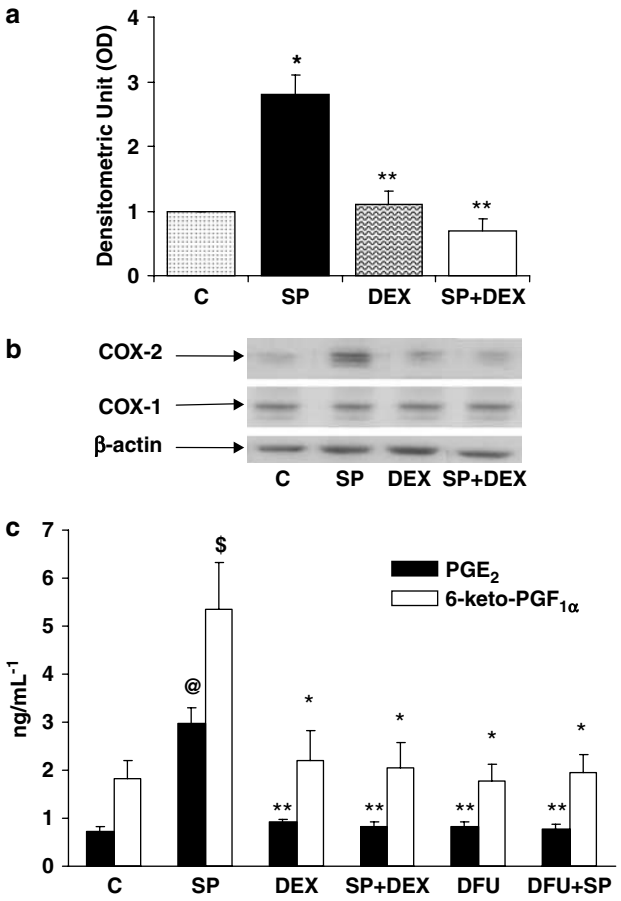

Figure 5 Pharmacological modulation of SP-induced COX-2 expression and activity. (a) HUVEC were stimulated with SP or DEX (100 nM) for 20 h or coincubated with SP and DEX for 20 h. Cells were processed for Western blot analysis as described in the Methods section. Results are expressed in densitometric units (OD) with control = 1 (means ± s.e. of five separate experiments). Data were analysed by one-way analysis of variance (ANOVA) and the Student–Newman–Keuls test (*$P<0.05$ *versus* control, **$P<0.05$ *versus* SP). (b) Immunoblot of one experiment representing COX-2 protein expression in the presence of 100 nM SP and DEX. (c) HUVEC were stimulated as described above and also in the presence of DFU (10 $\propto$M) or coincubated with SP and DFU for 20 h. 6-keto-$PGF_{1\alpha}$ or PGE_2 were analysed for PG release determination as described in the Methods section. The results are expressed as means ± s.e. of three independent experiments performed in duplicate. Data were analysed by one-way analysis of variance (ANOVA) and the Student–Newman–Keuls test ($ and @$P<0.01$ *versus* control for 6-keto-$PGF_{1\alpha}$ and PGE_2 * and **$P<0.05$ *versus* SP for 6-keto-$PGF_{1\alpha}$ and PGE2, respectively).

molecular weight of 53 and 44 kDa (Figure 4b) but no NK_3 expression (data not shown).

Pharmacological modulation of SP-induced COX-2 expression and activity

As there is ample evidence that glucocorticoids interfere with PG synthesis, acting at different levels of COX-2 gene regulation, we investigated the effect of DEX on SP-induced COX-2 expression. As is shown in Figure 5a and b, 100 nM DEX reduced COX-2 expression in HUVEC stimulated by 100 nM SP, but had no significant effect on untreated cells. DEX produced almost no change in COX-1 expression; it caused complete inhibition of 6-keto-$PGF_{1\alpha}$ and PGE_2 synthesis in HUVEC challenged by SP (Figure 5c) but did not affect PG synthesis in untreated cells. To investigate the contributions of different COX isoforms to endogenously-derived PG synthesis, we analysed the selective inhibition of COX-2 activities utilising an experimental selective COX-2 inhibitor, DFU. As shown in Figure 5c, treatment with DFU almost completely inhibited SP-induced PG synthesis, suggesting that the synthesis of both PGI_2 and PGE_2 induced by SP in HUVEC is predominantly COX-2 derived.

We further evaluated SP receptor involvement by assessing the effects of receptor agonists on SP-induced COX-2 expression and PG release in HUVEC in the same experi-

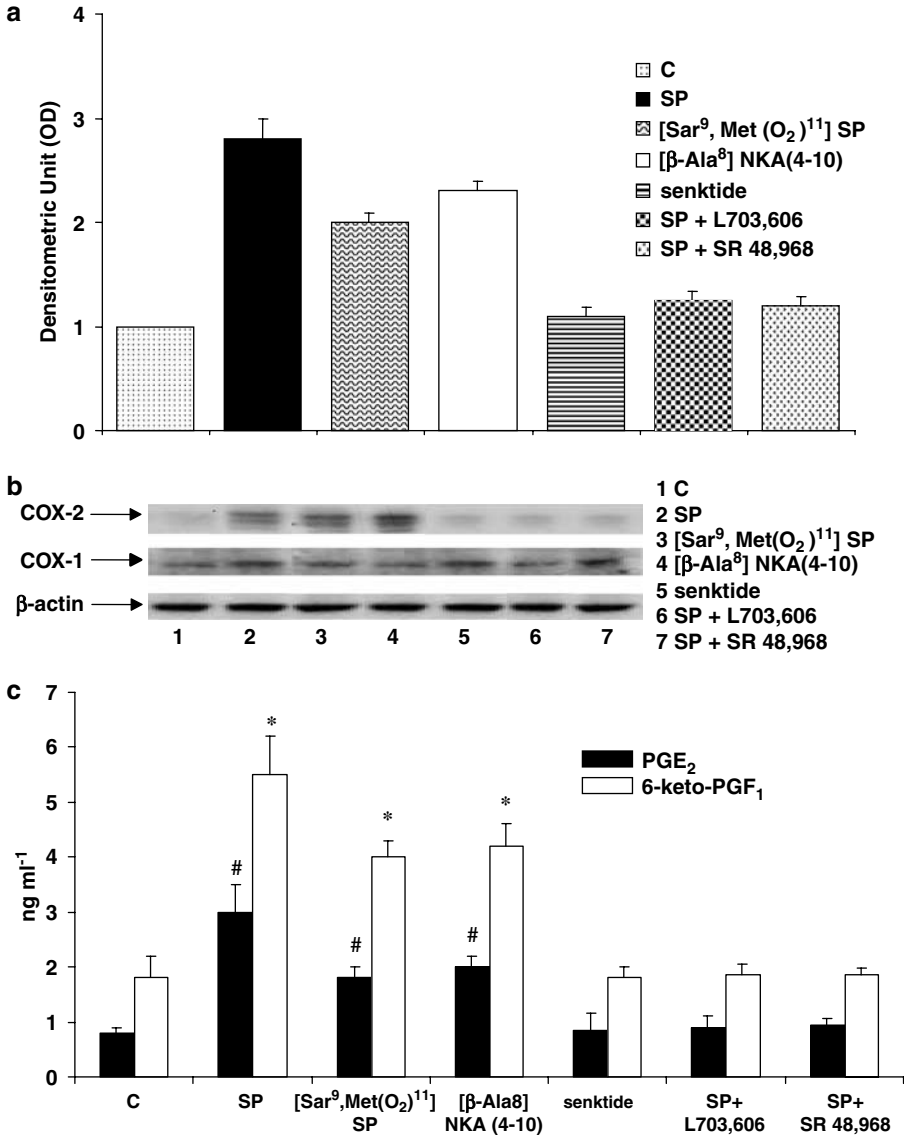

Figure 6 Effect of tachykinin receptor agonists and antagonists on COX-2 expression and on PG production in HUVEC. (a) HUVEC were stimulated with 100 nM SP or [Sar^9, $Met(O_2)^{11}$]SP or [β-Ala^8] NKA(4–10) or senktide or SP plus L703,606 or SP plus SR 48,968 for 20 h and processed for Western blot analysis as described in the Methods section. Results are expressed in densitometric units (OD) with control = 1 (means ± s.e. of four separate experiments). Data were analysed by one-way analysis of variance (ANOVA) and the Dunnet test (*$P<0.01$ *versus* control). (b) Immunoblot of one experiment representing COX-2 protein expression in the presence of SP-agonists or antagonists. (c) HUVEC were stimulated as described above. 6-keto-$PGF_{1\alpha}$ or PGE_2 were analysed for PG release determination as described in the Methods section. The results are expressed as means ± s.e. of three independent experiments performed in duplicate. Data were analysed by one-way analysis of variance (ANOVA) and the Dunnet test (* and $^{\#}P<0.01$ *versus* control).

mental conditions (100 nM, 20 h incubation): the NK_1 receptor agonist [Sar^9, $Met(O_2)^{11}$]SP, the NK_2 receptor agonist [β-Ala^8] NKA(4–10) and the NK_3 receptor agonist senktide. Figure 6a and b show that [Sar^9, $Met(O_2)^{11}$]SP and [β-Ala^8] NKA(4–10) induced increases in COX-2 protein expression similar to those obtained with SP, whereas senktide was ineffective in all cases. Figure 6c shows that 6-keto-$PGF_{1\alpha}$ and PGE_2 synthesis were statistically different from controls when HUVEC were incubated for 20 h with 100 nM SP, [Sar^9, $Met(O_2)^{11}$]SP or [β-Ala^8] NKA(4–10), but not in the presence of senktide. When HUVEC were incubated with SP in the presence of L703,606 (100 nM; an NK_1 selective antagonist) or SR 48,968 (100 nM; an NK_2 selective antagonist), neither COX-2 expression nor PG production were affected. We ran tests with the two selective antagonists alone, evidencing and found them to have no effect on COX-2 expression or PG production on HUVEC *per se* (data not shown).

Discussion

The results show that SP can induce COX-2 expression and PG release in HUVEC; these effects are receptor mediated, as NK_1 and NK_2 receptors are present on HUVEC and specific SP agonists and antagonists modulate these phenomenon.

Nonstimulated HUVEC expressed low or undetectable COX-2 levels, across all umbilical cords utilised. SP increased COX-2 expression in all cell cultures tested, producing a reproducible two- to three-fold increase over baseline. In no case was COX-1 affected by SP treatment. Peak effects of SP occurred at 100 nM and 20 h incubation. This incubation time is in agreement with those recorded in the presence of other endothelial stimuli, such as IL-1α and β or TNF-α (Caughey *et al*., 2001; Eligini *et al*., 2001). Dose–response experiments displayed a bell-shaped curve, with the values measured at 1 μM SP close to those recorded at 1 nM. The range of SP concentrations tested here compares with that reported to evoke COX-2 expression in other cell types (Jallat-Daloz *et al*., 2001; Tokuda *et al*., 2004). A bell-shaped dose–response curve has been found for SP in experimental models that measured the effect of the neuropeptide on the adhesion of splenocytes or PMNs to ECs (Vishwanath & Mukherjee, 1996; Dianzani *et al*., 2003). Higher concentrations were not investigated because the densitometric values recorded at 1 μM SP were close to control values. Furthermore, SP concentrations above the micromolar may recruit cell-activation mechanisms other than those involving specific receptor interactions (Dianzani *et al*., 2003).

Eligini *et al*. (2001) showed that the exposure of HUVEC to the NO-donor SIN-1 causes COX-2 expression without any corresponding increase in PG release. This unusual result prompted us to evaluate whether COX-2 expression induced by SP might translate into increased secretion of PGs by the cells. In our experimental model, induction of COX-2 following exposure to SP resulted in an increased release of PGI_2 and PGE_2 (respectively, four- and six-fold over control values), with PGI_2 values above those of PGE_2 throughout the tested range of concentrations and incubation times. The ability of DFU (which selectively inhibits COX-2 (>300 fold) *versus* COX-1) (Riendeau *et al*., 1997) to bring the PGI_2 and PGE_2 release induced by SP down to control values suggests that the increase in PG release mostly depends on varied levels of COX-2 induced by the neuropeptide.

Here, we show that SP activates p38 and p42/44 MAPKs, which is in agreement with studies showing SP to induce p38 and p42/44 activation in different cell types, with a similar time course (Fiebich *et al*., 2000; Koon *et al*., 2004). The specific involvement of p38 and p42/44 MAPK activation in SP-induced COX-2 expression is further supported by data showing that addition to HUVEC of the p38 MAPK inhibitor SB202190, or of the MEK1 inhibitor PD98059, significantly reduced COX-2 expression but had no effect on COX-1. It is known that transcriptional regulation of the COX-2 gene occurs *via* MAPK activation (Guan *et al*., 1998). COX-2 induction by stimuli other than SP has been reported to be mediated by both the p38 and the p42/44 MAPK pathways in various cell types, including HUVEC (Caughey *et al*., 2001). It has also been shown that the MAPK pathway is activated by SP in different cell types (Fiebich *et al*., 2000; Yang *et al*., 2002; Koon *et al*., 2004). Our results, which correlate the ability of SP to induce COX-2 expression in HUVEC with the activation of p38 and p42/44 MAPKs, are thus in agreement with data obtained in other cell types and provide the first demonstration of this mechanism in HUVEC.

The COX-2 gene is a target of glucocorticoid action, and cytokine-induced COX-2 expression is strongly repressed by DEX in several experimental systems (Newton *et al*., 1998; Uracz *et al*., 2002). Some mechanisms whereby DEX acts on trascriptional repression include transrepression of glucocorticoid elements (Drouin *et al*., 1993), repression of AP-1 dependent transactivation (Konig *et al*., 1992) and upregulation of NF-κB inhibitors (Uracz *et al*., 2002). SP treatment enhanced LPS-induced NF-κB binding activity in human dental pulp cultures (Tokuda *et al*., 2004) and activated NF-κB gene expression in human dermal microvascular cells (Quinlan *et al*., 1999). In our study we found that DEX completely suppressed COX-2 expression and activity in the presence of SP, suggesting SP may also modulate COX-2 expression at the nuclear level in HUVEC.

To evaluate the involvement of SP receptor(s) in COX-2 expression and PG production, we first ran tests to identify the presence of SP receptors on HUVEC: through RT–PCR and Western blot techniques we demonstrated the presence of NK_1 and NK_2 but not NK_3 receptors. We then analysed the effects of selective SP receptor agonists and antagonists on SP-mediated COX-2 induction and on PG release, so as to determine the single receptor involvement. SP effects on COX-2 modulation were mimicked only by the selective NK_1 and NK_2 receptor agonists [Sar^9, $Met(O_2)^{11}$]SP and [β-Ala^8] NKA(4–10), but not by the selective NK_3 receptor agonist senktide. Furthermore, the selective NK_1 and NK_2 antagonists, respectively L703,606 and SR 48,968, demonstrated their ability to revert SP-mediated effects not only on COX-2 expression but also on PG production. This finding of the involvement of both NK_1 and NK_2 receptors in mediating the effects of SP is in keeping with our previous finding that PMN adhesion to HUVEC evoked by SP depends on the activation of these two receptors (Dianzani *et al*., 2003). In both experimental models, activation of either NK_1 or NK_2 receptors by selective agonists was sufficient to evoke a response of the same order as that evoked by an equimolar concentration of the natural ligand, demonstrating that activation of only one receptor type is sufficient to evoke the

same response by SP. On the other hand, the absence of NK_3 receptor in HUVEC is in agreement with reports that this receptor is mainly found in the central nervous system and is absent or present only in small amounts in peripheral tissues (Patak *et al*., 2003).

SP ability to induce the release of PG from HUVEC was reported by Alhenc-Gelas *et al*. (1982) and by Marceau *et al*. (1989), without demonstrating the expression of COX proteins. Our model comprised incubation for 20 h, whereas Marceau *et al*. (1989) employed only 15 min; they plotted a shallow dose–response curve in the 10 nM–10 μM range, with maximum increase of PGI_2 release at 10 μM (two- to three-fold control values). Our dose–response curve is quite different. Another important difference between the two sets of experiments is that Marceau *et al*. (1989) found the NK_2 agonist to have no effect. The reasons for these discrepancies cannot easily be identified, but may be related to different experimental conditions, particularly the incubation time.

The release of neuropeptides, such as SP, by the peripheral sensory fibres may act as an efferent signal to facilitate cell recruitment and cytokine release at inflammatory sites (Dianzani *et al*., 2001). The close proximity of these fibres to capillaries, for instance in the skin, stresses the functional interaction between nerve fibres and endothelial cells. SP released by unmyelinated sensory nerve C fibres evokes a number of endothelial responses, such as proliferation, angiogenesis, shape change (rounding) (Ziche *et al*., 1994; Seegers *et al*., 2003) and increased vascular permeability (Harrison & Geppetti, 2001). Here we add a previously undetected effect: the upregulation of COX-2 and PG release in HUVEC by SP. Cross-talk among peripheral sensory fibres, leukocytes and EC at the site of injury mediate the development of neurogenic inflammation. The ability of SP to increase the expression of COX-2 in EC highlights the relationship between neuropeptides and the endothelium in the development of this pathophysiological event.

This research was supported by Turin University funding (ex-60%). We thank the Anatomo-Pathology Unit, Directed by Dr Cardesi and the Obstetrics and Gynecology Unit, directed by Dr Carnino, Martini Hospital, Turin, for providing human umbilical cords.

References

ALHENC-GELAS, F., TSAI, S.J., CALLAHAN, K.S., CAMPBELL, W.B. & JOHNSON, A.R. (1982). Stimulation of prostaglandin formation by vasoactive mediators in cultured human endothelial cells. *Prostaglandins,* **24,** 723–742.

BRUNELLESCHI, S., TARLI, S., GIOTTI, A. & FANTOZZI, R. (1991). Priming effects of mammalian tachykinins on human neutrophils. *Life Sci.,* **48,** PL1–PL5.

CAUGHEY, G.E., CLELAND, L.G., PENGLIS, P.S., GAMBLE, J.R. & JAMES, M.J. (2001). Roles of cyclooxygenase (COX)-1 and COX-2 in prostanoid production by human endothelial cells selective up-regulation of prostacyclin synthesis by COX-2. *J. Immunol.,* **167,** 2831–2838.

DAVIES, S.P., REDDY, H., CAIVANO, M. & COHEN, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.,* **351,** 95–105.

DIANZANI, C., LOMBARDI, G., COLLINO, M., FERRARA, C., CASSONE, M.C. & FANTOZZI, R. (2001). Priming effects of substance P on calcium changes evoked by interleukin-8 in human neutrophils. *J. Leukoc. Biol.,* **69,** 1013–1018.

DIANZANI, C., COLLINO, M., LOMBARDI, G., GARBARINO, G. & FANTOZZI, R. (2003). Substance P increases neutrophil adhesion to human umbilical vein endothelial cells. *Br. J. Pharmacol.,* **139,** 1103–1110.

DROUIN, J., SUN, Y.L., CHAMBERLAND, M., GAUTHIER, Y., DE LEAN, A., NEMER, M. & SCHMIDT, T.J. (1993). Novel glucocorticoid receptor complex with DNA element of the hormone-repressed POMC gene. *EMBO J.,* **12,** 145–156.

ELIGINI, S., HABIB, A., LEBRET, M., CREMINON, C., LEVY-TOLEDANO, S. & MACLOUF, J. (2001). Induction of cyclo-oxygenase-2 in human endothelial cells by SIN-1 in the absence of prostaglandin production. *Br. J. Pharmacol.,* **133,** 1163–1171.

FIEBICH, B.L., SCHLEICHER, S., BUTCHER, R.D., CRAIG, A. & LIEB, K. (2000). The neuropeptide substance P activates p38 mitogen-activated protein kinase resulting in IL-6 expression independently from NF-kappa B. *J. Immunol.,* **165,** 5606–5611.

FOREMAN, J.C. & JORDAN, C.C. (1984). Neurogenic inflammation. *Trends Pharmacol. Sci.,* **5,** 116–119.

GUAN, Z., BUCKMAN, S.Y., MILLER, B.W., SPRINGER, L.D. & MORRISON, A.R. (1998). Interleukin-1beta-induced cyclooxygenase-2 expression requires activation of both c-Jun NH2-terminal kinase and p38 MAPK signal pathways in rat renal mesangial cells. *J. Biol. Chem.,* **273,** 28670–28676.

HARRISON, S. & GEPPETTI, P. (2001). Substance P. *Int. J. Biochem. Cell. Biol.,* **33,** 555–576.

HIRAI, K., EZUMI, Y., NISHIDA, E., UCHIYAMA, T. & TAKAYAMA, H 1999. Comparative study of vanadate- and phorbol ester-induced cyclo-oxygenase-2 expression in human endothelial cells. *Thromb. Haemost.,* **82,** 1545–1552.

JAFFE, E.A., NACHMAN, R.L., BECKER, C.G. & MINICK, C.R. (1973). Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest.,* **52,** 2745–2756.

JALLAT-DALOZ, I., COGNARD, J.L., BADET, J.M. & REGNARE, J. (2001). Neural-epithelial cell interplay: *in vitro* evidence that vagal mediators increase PGE2 production by human nasal epithelial cells. *Allergy Asthma Proc.,* **22,** 17–23.

JOOS, G.F. & PAUWELS, R.A. (2000). Pro-inflammatory effects of substance P: new perspectives for the treatment of airway diseases? *Trends Pharmacol. Sci.,* **21,** 131–133.

KONIG, H., PONTA, H., RAHMSDORF, H.J. & HERRLICH, P. (1992). Interference between pathway-specific transcription factors: glucocorticoids antagonize phorbol ester-induced AP-1 activity without altering AP-1 site occupation *in vivo*. *EMBO J.,* **11,** 2241–2246.

KOON, H.W., ZHAO, D., NA, X., MOYER, M.P. & POTHOULAKIS, C. (2004). Metalloproteinases and transforming growth factor-alpha mediate substance P-induced mitogen-activated protein kinase activation and proliferation in human colonocytes. *J. Biol. Chem.,* **279,** 45519–45527.

KOYAMA, Y., MIZOBATA, T., YAMAMOTO, N., HASHIMOTO, H., MATSUDA, T. & BABA, A. (1999). Endothelins stimulate expression of cyclooxygenase 2 in rat cultured astrocytes. *J. Neurochem.,* **73,** 1004–1011.

KYRIAKIS, J.M. & AVRUCH, J. (2001). Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol. Rev.,* **81,** 807–869.

MARCEAU, F., TREMBAY, B., COUTURE, R. & REGOLI, D. (1989). Prostacyclin release induced by neurokinins in cultured human endothelial cells. *Can. J. Physiol. Pharmacol.,* **67,** 159–162.

MARRIOTT, D.R., WILKIN, G.P. & WOOD, J.N. (1991). Substance P-induced release of prostaglandins from astrocytes: regional specialisation and correlation with phosphoinositol metabolism. *J. Neurochem.,* **56,** 259–265.

NEWTON, R., SEYBOLD, J., KUITERT, L.M., BERGMANN, M. & BARNES, P.J. (1998). Repression of cyclooxygenase-2 and prostaglandin E2 release by dexamethasone occurs by transcriptional and post-transcriptional mechanisms involving loss of polyadenylated mRNA. *Biol. Chem.,* **273,** 32312–32321.

PAGE, N.M. & BELL, N.J. (2002). The human tachykinin NK1 (short form) and tachykinin NK4 receptor: a reappraisal. *Eur. J. Pharmacol.,* **437,** 27–30.

PATACCHINI, R. & MAGGI, C.A. (1995). Tachykinin receptors and receptor subtypes. *Arch. Int. Pharmacodyn. Ther.,* **329,** 161–184.

PATAK, E., CANDENAS, M.L., PENNEFATHER, J.N., ZICCONE, S., LILLEV, A., MARTIN, J.D., FLORES, C., MANTECON, A.G., STORY, M.E. & PINTO, F.M. (2003). Tachykinins and tachykinin receptors in human uterus. *Br. J. Pharmacol.,* **139,** 523–532.

QUINLAN, K.L., NAIK, S.M., CANNON, G., ARMSTRONG, C.A., BUNNETT, N.W., ANSEL, J.C. & CAUGHMAN, S.W. (1999). Substance P activates coincident NF-AT- and NF-kappa B dependent adhesion molecule gene expression in microvascular endothelial cells through intracellular calcium mobilization. *J. Immunol.,* **163,** 5656–5665.

REGOLI, D., BOUDON, A. & FAUCHERE, J.L. (1994). Receptors and antagonists for substance P and related peptides. *Pharmacol. Rev.,* **46,** 551–599.

RIENDEAU, D., PERCIVAL, M.D., BOYCE, S., BRIDEAU, C., CHARLESON, S., CROMLISH, W., ETHIER, D., EVANS, J., FALGUEYRET, J.P., FORD-HUTCHINSON, A.W., GORDON, R., GREIG, G., GRESSER, M., GUAY, J., KARGMAN, S., LEGER, S., MANCINI, J.A., O'NEILL, G., OUELLET, M., RODGER, I.W., THERIEN, M., WANG, Z., WEBB, J.K., WONG, E., XU, L., YOUNG, R.N., ZAMBONI, R., PRASIT, P. & CHAN, C.C. (1997). Biochemical and pharmacological profile of a tetrasubstituted furanone as a highly selective COX-2 inhibitor. *Br. J. Pharmacol.,* **121,** 105–117.

ROZEN, S. & SKALETSKY, H.J. (2000). Primer3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. eds. Krawetz, S. & Misener, S. pp. 365–386. Totowa NJ: Humana Press.

SEEGERS, H.C., HOOD, V.C., KIDD, B.L., CRUWYS, S.C. & WALSH, D.A. (2003). Enhancement of angiogenesis by endogenous substance P release and neurokinin-1 receptors during neurogenic inflammation. *J. Pharmacol. Exp. Ther.,* **306,** 8–12.

TOKUDA, M., MIYAMOTO, R., NAGAOKA, S. & TORII, M. (2004). Substance P enhances expression of lipopolysaccharide-induced inflammatory factors in dental pulp cells. *J. Endod.,* **30,** 770–773.

URACZ, W., URACZ, D., OLSZANECKI, R. & GRYGLEWSKI, R.J. (2002). Interleukin 1beta induces functional prostaglandin E synthase in cultured human umbilical vein endothelial cells. *Physiol. Pharmacol.,* **53,** 643–654.

VANE, J.R., BAKHLE, Y.S. & BOTTING, R.M. (1998). Cyclooxygenases 1 and 2. *Annu. Rev. Pharmacol. Toxicol.,* **38,** 97–120.

VISHWANATH, R. & MUKHERJEE, R. (1996). Substance P promotes lymphocyte-endothelial cell adhesion preferentially *via* LFA-1/ICAM-1 interactions. *Neuroimmunol.,* **71,** 163–171.

YANG, C.M., HSIAO, L.D., CHIEN, C.S., LIN, C.C., LUO, S.F. & WANG, C.C. (2002). Substance P-induced activation of p42/44 mitogen-activated protein kinase associated with cell proliferation in human tracheal smooth muscle cells. *Cell Signal.,* **14,** 913–923.

ZICHE, M., MORBIDELLI, L., MASINI, E., AMERINI, S., GRANGER, H.J., MAGGI, C.A., GEPPETTI, P. & LEDDA, F. (1994). Nitric oxide mediates angiogenesis *in vivo* and endothelial cell growth and migration *in vitro* promoted by substance P. *J. Clin. Invest.,* **94,** 2036–2044.

(Received December 6, 2005
Accepted December 14, 2005
Published online 28 January 2006)